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Naghdi, Elahe; De Malsche, Wim

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Overloading behavior of fenoprofen and naproxen as two model compounds on a non-porous silicon pillar array column

Elahe Naghdi[1,2], Wim De Malsche[1*]

1. µFlow group, Department of Chemical Engineering, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium.
2. Faculty of Chemistry, Shahid Beheshti University, G.C., Tehran, I.R. Iran.

(*) corresponding author

Pleinlaan 2, B-1050, Brussels, Belgium

Tel.: +32 (0) 2 629 3781, Fax.; +32-2 629 3248, E-mail: Wim.De.Malsche@vub.be

Abstract

In this study, the adsorption behavior of naproxen and fenoprofen as two model compounds on a non-porous pillar array column (NPAC) was investigated under reverse phase liquid chromatography conditions. Band profiles of both analytes were recorded in overloaded concentrations using 30% methanol/water (v/v) as the mobile phase. Breakthrough experiments under the same chromatographic condition were carried out to measure the adsorption isotherms. Single-component adsorption isotherm data were acquired by frontal analysis for each analyte.. The isotherms were found to be concave upward and downward for naproxen and fenoprofen, respectively. To find the best agreement between the experimental data points and the adsorption isotherm models, the obtained isotherms were modeled using several isotherm models. The Langmuir-Freundlich and anti-Langmuir models provided the best fitting for fenoprofen and naproxen, respectively. The solute and stationary phase properties determine the appropriate model. Adsorbate–adsorbate interaction is important in the case of naproxen, while the adsorbate-adsorbent (stationary phase) plays the main role in retention of fenoprofen on the NPAC. The validity of the selected isotherm models were checked comparing calculated and experimental band profiles and plate heights. An excellent agreement was observed for the whole concentration range of both analytes, which confirmed the accuracy of the selected models.

Keywords: Adsorption isotherm; Anti-Langmuir model; Equilibrium-dispersive model; Langmuir-Freundlich model; Non-porous pillar array;

1. Introduction

Preparative chromatography is one of the most powerful separation processes for the purification of valuable and pharmaceuticals products [1-3]. Chromatography is often preferred because the process allows for both high yields and purities.

The optimization of a chromatographic separation process at a preparative scale is generally a complex task and requires a careful selection of the operation conditions. Therefore, ideally, one of the first steps in studying this process is the determination of the equilibrium adsorption isotherms of the analytes. This allows gaining insight in the retention mechanism and to predict and evaluate the production rate recoveries and eventually separation costs [4].

Purification of large sample amounts by preparative high performance liquid chromatography (HPLC) is possible using two methods: 1) scale-up of the analytical system, 2) working at maximal concentration. Scale-up of the analytical system implies using a larger column and higher flow rate of mobile phase.

Column overloading can occur through (a) volume overloading or (b) concentration overloading. In concentration overload, a small volume of the sample is injected, but its concentration exceeds the linear range of the adsorption isotherm. As a result, the band profile broadens and becomes unsymmetrical. Concentration overloading has been reported to purification of various compounds for which there is an interest to purify, as. e.g. phosphorothioated oligonucleotides [5], anthocyanin glucoside cinnamic ester isomers [6] and peptides [7].

The adsorption isotherm at equilibrium between the amounts adsorbed in the stationary phase and the concentration in the mobile phase determines the direction of the band asymmetry, either as a fronting or tailing shape. The overload band profiles and related the adsorption isotherms in reverse phase liquid chromatography (RP-LC) conditions using packed [8,9] and monolithic [10,11] columns have been studied to understand of retention and overloading mechanisms.

Pillar array columns (PACs) have been introduced as an alternative column format to packed bed and monoliths in analytical liquid chromatography (LC) and have shown to yield high peak capacities and column efficiencies. Recently, porous PACs have been successfully used to separation of chiral and non-chiral compounds [12-14]. From the point of view of minimizing

dispersion (and peak capacity) however, non-porous support structures are fundamentally superior, and typically produce a double plate count of what is achieved for the same non-porous column [15], when working at low concentration is possible (e.g. due to detection sensitivity constraints). When the analytes are large (e.g. DNA or RNA strands or large proteins), it is not desirable to have the large analyte molecules entering similarly sized pores, which leads to excessive dispersion. Using non-porous silicon pillars allowed for high resolution separation of DNA strands in ion-pair reversed phase chromatography mode [16].

Several applications with non-porous PACs (NPACs) using small molecules have been reported as well [17, 18]. Even for analytical purposes it is instrumental to understand overloading behavior, as detection limits (or statistical considerations) generally requires for at least a few components to work at overloading conditions. In the present study, we investigated the application of NPAC in concentration overloading conditions. Two commonly used non-steroidal anti-inflammatory drugs (naproxen and fenoprofen) were selected as representative analytes. To gain a better understanding in the general retention mechanism of these two compounds in RP-LC using NPACs, the single-component adsorption isotherms of the analytes were measured by frontal analysis. The adsorption isotherms were modeled by anti-Langmuir and Langmuir-Freundlich models for naproxen and fenoprofen, respectively. This information is subsequently used to estimate band profiles at given concentrations.

2. Theory

2.1. Determination of the single-component adsorption isotherms by frontal analysis method

Frontal analysis (FA) is an accurate method [19] that allows for in-situ measurement (in the intact separation column), and is the method of the choice when very small amounts of stationary phase are available (or when the stationary phase material is embedded and cannot be separately prepared or isolated, as in the case of PACs). In FA, the breakthrough curves are recorded by the successive replacement of the stream of mobile phase through the column with streams of sample solutions of increasing concentrations.

Mass conservation of the solute between the times when the new solution enters the column and when the plateau concentration is reached allows for the calculation of the mass of solute retained

in the column at equilibrium (m^*_t) with the sample concentration in mobile phase (C). This mass is measured by integrating the breakthrough curve [20]:

$$m^*_t = CV_{eq} \quad (1)$$

where V_{eq} indicates the elution volume of the equivalent area of the solute.

The amount of adsorbed solute on the stationary phase (q^*) at equilibrium with a sample concentration in the mobile phase is given by:

$$q^* = (m^*_t - C\,V_0)/V_a \quad (2)$$

V_0 and V_a show the column void volume and the volume of stationary phase, respectively. V_0 was determined from the elution time of uracil as a non-retained compound. The information on the adsorption behavior is captured in the set of pairs q^* and C.

2.2. Modeling of the single-component adsorption isotherms

After the experimental evaluation of the adsorption data, an isotherm model is useful to interpret the adsorption behavior and the overall chromatographic process. Langmuir-Freundlich and the anti-Langmuir models were used to describe the adsorption isotherm of fenoprofen and naproxen, respectively. The Freundlich-Langmuir isotherm equation is given by [21]:

$$q^* = q_s\,K\,C^{1/n}/1 + K\,C^{1/n} \quad (3)$$

where q_s, K and n indicate the column saturation capacity, binding constant and heterogeneity index, respectively.

The anti-Langmuir isotherm equation is given by [22]:

$$q^* = aC/(1-bC) \quad (4)$$

with a and b numerical coefficients.

2.3. Modeling of band profiles in HPLC

The overloaded band profiles of naproxen and fenoprofen were calculated using the equilibrium-dispersive (ED) model of chromatography. In this model, the mass balance equation for a single component is written:

$$\partial C/\partial t + u(\partial C/\partial z) + F(\partial q^*/\partial t) = D_a (\partial^2 C/\partial z^2) \quad (5)$$

where q^* and C are the stationary and mobile phase concentrations of the adsorbate at equilibrium, respectively, t the time z the distance along the column, D_a the axial dispersion coefficient u the linear mobile phase velocity, F the phase ratio defined by = V_a/V_0. q^* which is related to C by the isotherm equation [23].

The following initial and boundary conditions are used to solve equation 5.

At t = 0, the concentration of the solute in the column is equal to zero:

$$C(0, z) = 0 \quad (6)$$

The boundary condition at the column inlet is defined as:

$$C(t,0) = C_i \quad \text{for} \quad 0<t<t_p \quad \text{and} \quad C(t,0) = 0 \quad \text{for } t>t_p \quad (7)$$

with t_p the injection time of a rectangular injection plug.

The boundary condition at the column outlet is:

$$(\partial C/\partial z) = 0 \qquad z = L \text{ and } t>0 \quad (8)$$

The ED model was solved using the finite difference method [24].

2.5. Calculations of the chromatographic figures of merit

The peak asymmetry factor is a measurement of the peak tailing or fronting. The statistical moments are used for calculation of the asymmetry factor (As):

$$As = (\mu_1 - t_R)/\sqrt{\mu_2} \quad (9)$$

with t_R the retention time of the peak, μ_1 the first statistical moment and μ_2 the second statistical moment. For tailing peaks, the asymmetry factor is positive (As>0) while negative asymmetry (As<0) indicates fronting peaks.

The theoretical plate height (H) was calculated using the following equations:

$$N = t_R^2/\mu_2 \quad (10)$$

$$H = L/N \quad (11)$$

with N and L theoretical plate numbers and the column length, respectively.

3. Experimental

3.1. Material

Fenoprofen and uracil were purchased from Sigma-Aldrich (St. Louis, MO, USA). Naproxen reference standard was kindly provided by Temad Company (Tehran, Iran). Anhydrous toluene was purchased from Sigma-Aldrich (Overijse, Belgium). Octadecyldimethyl-N,N dimethylaminosilane (ODS-DMA) was purchased from ChemPur Feinchemikalien und Forschungsbedarf GmbH (Karlsruhe, Germany). Purified water from a Milli-Q reagent water system (Billerica, MA, USA) was used to prepare mobile phase and reagent solutions. LC/MS grade methanol and acetonitrile were also obtained from Biosolve (Valkenswaard, Netherland).

3.2. Apparatus

A Thermo Fisher Scientific Ultimate 3000 nano-LC system (Germering, Germany) equipped with a membrane degasser, LPG-3600 pump, FLM-3200 column oven, and a VWD-3400 RS UV-detector system was used for chromatography evaluation. The UV system was set at 215 nm for detection of fenoprofen and 230 nm for detection of naproxen. A 4 nl sample injector obtained from Valco (Schenkon, Switzerland) was employed for injection of samples. Data collection and processing were done by the Chromeleon software.

3.3. NPAC fabrication procedure

A 20 cm long and 315 µm wide pillar-array channel (5 µm diameter pillars, inter-pillar distance 2.5 µm) was patterned using normal UV photolithography (photoresist, Olin 907-12), followed by a dry etching step (Adixen AMS100DE, Alcatel Vacuum Technology, Culemborg, The

Netherlands) to etch the 200 nm thick SiO_2 hard mask underneath. Next, the capillary channels were defined by subsequent mid-UV lithography, etching of the SiO_2 layer by a Bosch-type deep-reactive-ion etching step (Adixen AMS100SE) reaching a depth of 80 µm. After this, the resist was removed by oxygen plasma and nitric acid, and the pillars defined in the SiO_2 mask (and also the already defined and partly etched capillary groove) were subsequently Bosch etched to reach a depth of 18 µm (and the capillary channel a total depth of about 130 µm) [17].

Next, the columns were modified with C_{18} coating using ODS-DMA as described in detail previously [25]. Briefly, 10% ODS-DMA/toluene (v/v) was pumped through column at 60 ˚C for 24h and washed with toluene, 50 % acetonitrile/toluene (v/v), and then acetonitrile. A typical SEM image of the NPAC is indicated in Fig .1. The properties of the NPAC are summarized in table 1.

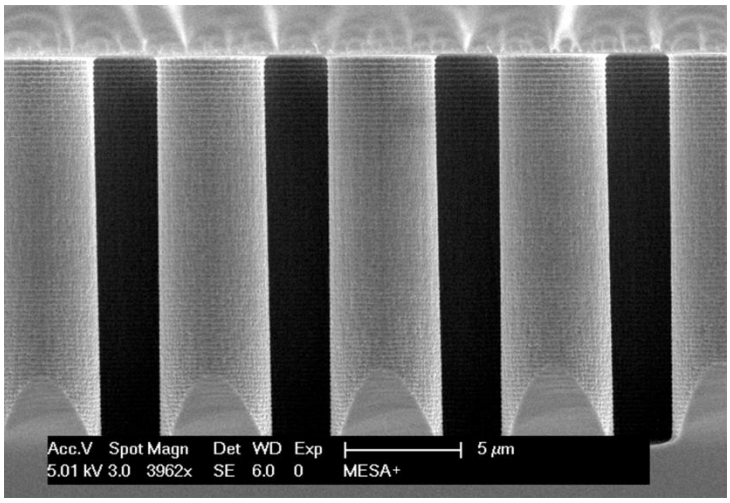

Fig .1. SEM picture of pillars comprising the NPAC

Table 1. The NPAC properties.

Column length	Channel width	Pillar type	Pillar height	Pillar diameter	Inter pillar distance
20 cm	315 µm	Cylindrical	18 µm	5 µm	2.50 µm

3.4. Measurements of the overloaded band profiles of naproxen and fenoprofen on NPAC

The standard stock solution of the chemicals was prepared in LC/MS grade methanol at a concentration of 1000 µg mL^{-1}. The solutions were stored at 4°C and heated to room temperature

before use. Sample containing naproxen and fenoprofen were prepared daily freshly via dilution of stock solution with the used mobile phase (methanol/water) to get adequate concentration. The overloading profiles of naproxen and fenoprofen were recorded at 230 and 215 nm, respectively.

3.5. Determination of the adsorption isotherms by FA

The appropriate chromatographic condition to obtain the adsorption isotherm was determined by the shape of the recorded band profile. 30 % methanol/water (v/v) as the mobile phase at a flow-rate of 1.0 µl/min and the column temperature at 35 ˚C was used which lead to fronting and tailing band shape for naproxen and fenoprofen, respectively.

Prior to the isotherm determinations, approximate values of the solubility of analytes were determined. A concentration of 100 ppm was selected for naproxen and 200 ppm for fenoprofen to avoid precipitation in the instrument. Two master solutions of each profen were prepared at 10 and 100% of the selected concentration. One pump of the HPLC instrument was used to deliver a flow of the pure mobile phase (30 % methanol/water (v/v)) and two other pumps pumped a flow of the 10% and 100% sample solutions into the NPAC. In case of fenoprofen, consecutive FA runs were performed starting from the lowest to the highest concentrations. The opposite direction was used in case of naproxen, i.e., the FA procedure started from the highest concentration and continued to the lowest concentration. The breakthrough curves were recorded with a sufficiently long time between each step (20 and 15 min for fenoprofen and naproxen, respectively) to allow for the complete re-equilibration of the column. The detector was set at 230 nm for naproxen and 215 nm for fenoprofen.

4. Results and discussion

4.1. Overloaded band (elution) profiles

The elution profile of overloaded analytes contains information about the stationary phase and the mobile phase.

Two set of samples containing different concentration of naproxen and fenoprofen were introduced into the NPAC and elution profiles were recorded. Different compositions of methanol/water (35-

15% methanol/water (v/v) were tested as the mobile phase). The temperature of the column was also varied (15-35 ˚C). Application of acetonitrile/water compositions resulted similar results.

A set of elution profiles was generated with different concentration of each analyte using 30 % methanol/water (v/v) as the mobile phase at the column temperature of 35 ˚C. Typical results are given in Fig.2.

As is common for overloading conditions [26, 27], we observed that the analytes band shapes were dependent on the chromatographic conditions.

While naproxen exhibits a fronting band shape in most cases studied in the present paper (35% methanol/water (v/v) at 15-35 ˚C (As = -0.80 – (-1.1)), 30 % methanol/water (v/v) at 15-35 ˚C (As= -0.11 – (-0.89)) and 25 % methanol/water (v/v) at 25-35 ˚C (As = -0.26 – (-0.29)), the fenoprofen peak is eluted with tailing in all studied conditions. The fronting peak of naproxen (see Fig. 2) is assumed to emerge from an interaction between naproxen molecules (next to a weaker interaction with the stationary phase) and the tailing peak of fenoprofen arises only from interactions between the fenoprofen molecules and the stationary phase.

Using 15% methanol/water (v/v) as the mobile phase caused tailing peak for naproxen at 15-30 ˚C (As = 0.65–0.50). This indicates that the interaction between naproxen molecules and the column surface is stronger than the interaction between naproxen molecules when a mobile phase with high water content is used. In fact, the higher water content in the mobile phase leads to a larger number of high-energy sites on the stationary phase [28]. As a result, more interaction between the analyte molecules and stationary phase surface is possible.

Naproxen is less retained than fenoprofen on the NPAC column. The elution order can be understood by the degree of hydrophobicity. Naproxen with a lower octanol/water distribution partition coefficient (log D = 2.99) is eluted faster than fenoprofen (log D = 3.65).

Figures 2a and 2b show that the band profiles of naproxen and fenoprofen are very different. NPAC shows fronting peak when overloaded by naproxen at a concentration > 25 ppm (As<0). Fig. 2a depicts an upward concave isotherm and the increase of the amount injected does not change the retention time significantly and leads to a few increase in the retention time of naproxen. This

observation can be explained by the anti-Langmuir adsorption behavior, where solute–solute interactions cause increased retention as the sample load increases [29].

In contrast, for more hydrophobic drug (fenoprofen) in overloading conditions (concentration > 50 ppm) the tailing behavior (As>0) was observed on NPAC. Fig. 2b corresponds to the concave downward isotherm and the peak has the appearance of a triangle. The retention times move a bit to shorter values with increasing sample size, as commonly observed [30-32]. Also, the extents of the tailing increase with more sample load.

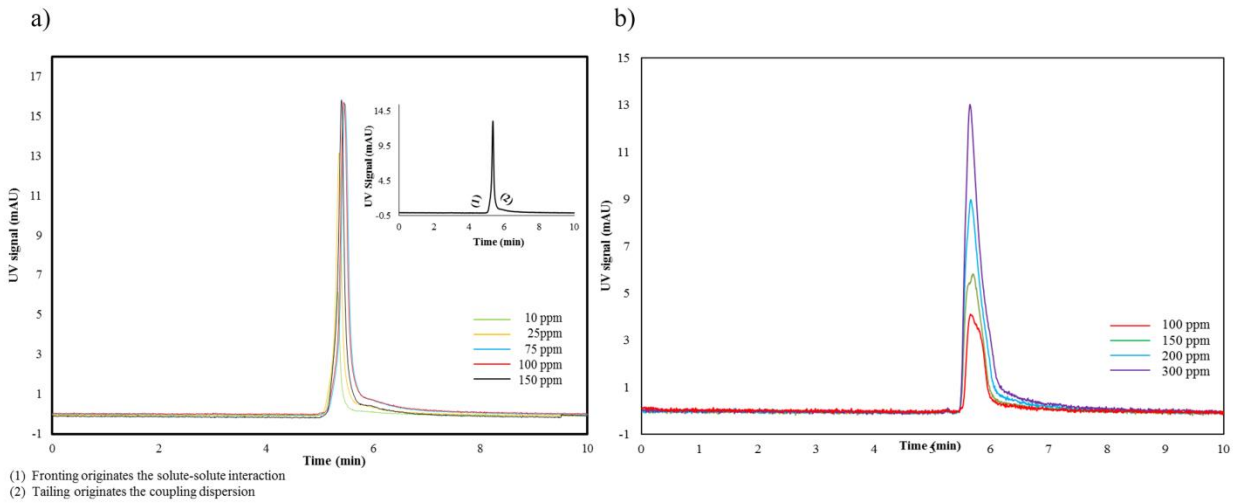

Fig. 2. Overloaded elution profiles of (a) naproxen at 230 nm and (b) fenoprofen at detected at 215 nm on the NPAC. Other chromatographic conditions are: mobile phase: 30% methanol/water (v/v), column temperature: 35 ˚C, mobile phase flow rate: 1.0 µl/min.

4.2. The adsorption isotherm of fenoprofen: Langmuir-Freundlich isotherm model

The position and shape of overloaded band profiles recorded in LC depends directly on the adsorption equilibrium isotherm of the compound between the stationary and the mobile phases. Therefore, to understand the behavior of the selected pharmaceutical analytes on NPAC in preparative RP-LC, adsorption isotherms of these analytes were measured. The obtained breakthrough curves are indicated in Fig. 1S and Fig. 2S.

The adsorption isotherm models most frequently encountered in liquid chromatography are of Langmuirian-type (Freundlich, Langmuir–Freundlich, Langmuir, Bi- Langmuir, Tri- Langmuir, Toth, etc.) isotherm model. Fig. 3 shows the isotherm curve for the experimental adsorption of

fenoprofen on NPAC. The experimental adsorption data was best fitted by the Freundlich-Langmuir model. The obtained isotherm parameters are listed in table 2. At the low concentrations, the Langmuir-Freundlich model reduces to the classical Freundlich and in the case of a homogeneous surface (N=1), it reduces to the classical Langmuir model [33].

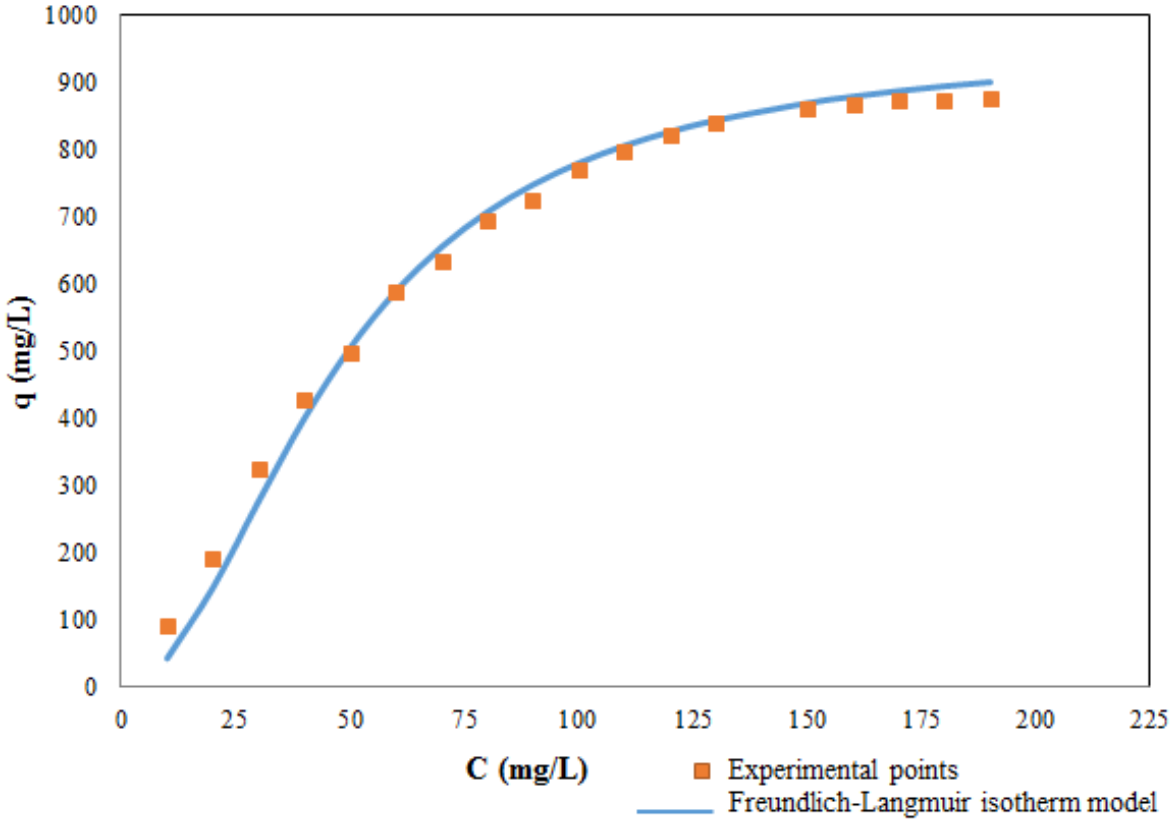

Fig. 3. The adsorption isotherm of fenoprofen on NPAC. Chromatography condition: mobile phase: 30% methanol/water (v/v), column temperature: 35 ˚C, mobile phase flow rate: 1.0 µl/min, detection: 215 nm.

Table 2. The adsorption isotherm parameters obtained by fitting the experimental FA data of the fenoprofen on NPAC using Freundlich-Langmuir model.

Column saturation capacity (mg/L)	Affinity constant (L/mg)	Heterogeneity parameter	Regression Coefficient (R^2)
958.19	$4.90*10^{-4}$	0.51	0.99

4.3. The adsorption isotherm of naproxen: Anti-Langmuir isotherm model

The fenoprofen isotherm is concave downward for the entire investigated concentration range, while the isotherm for naproxen is concave upward (Fig.4). The downward concave is mostly observed in liquid–solid or liquid-liquid equilibrium, however, the upward concave isotherm is less common in liquid chromatography [22].

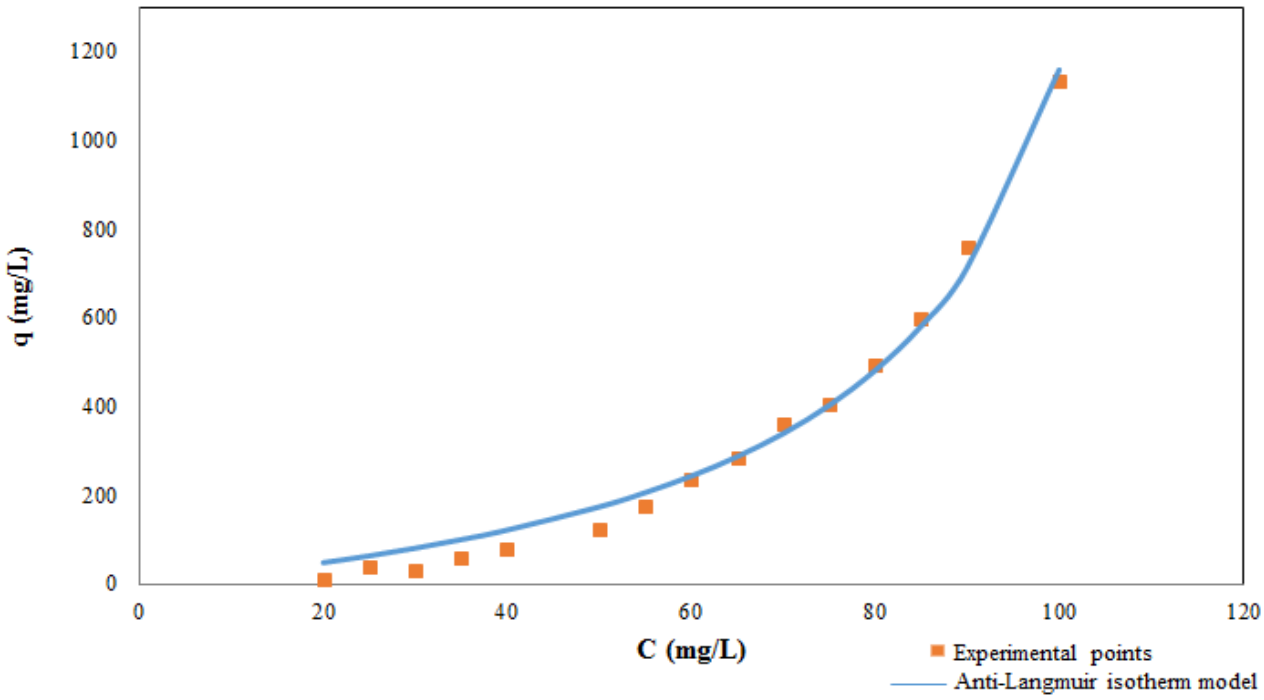

Fig.4. The adsorption isotherm of naproxen on NPAC. Chromatography condition: mobile phase: 30% methanol/water (v/v), column temperature: 35 ˚C, mobile phase flow rate:1.0 µl/min, detection: 230 nm.

In the upward concave situation, the concentration in the stationary phase at equilibrium increases faster than the concentration in the mobile phase. This means that a second molecule is more strongly adsorbed than a first one (or more general: adding a molecule to n molecules) which commonly indicates the existence of attractive interactions between the adsorbed molecules. Anti-Langmuir adsorption model describes the upward concave isotherms and accounts for the adsorption behavior due to solute–solute interactions [34]. The experimental data were fitted into several models and the best result was obtained by anti-Langmuir isotherm model. The parameters were obtained through nonlinear least-squares fitting (R^2 = 0.99, see table 3 for obtained parameters).

Table 3. The adsorption isotherm parameters obtained by fitting the experimental FA data of the naproxen on NPAC using anti-Langmuir model.

A	B	Regression Coefficient (R^2)
2.01	$8.22*10^{-3}$	0.99

4.4. Validation of isotherm models

According to Fig. 3, the isotherm model that accounts best for the adsorption data of fenoprofen on the NPAC is the Langmuir-Freundlich model. With the obtained parameters, it is also possible to predict the peak shape at a given concentration and mobile phase velocity. The experimentally obtained and predicted curves are depicted in Fig. 5 (dashed lines). The calculated profiles were derived using the equilibrium-dispersive model (see section 2.2). As can be noticed, the curves are in qualitative agreement. This result confirms that the selected model is adequate. There is some minor deviation near the baseline, which can most likely be predominantly attributed to coupling dispersion between the PAC outlet and the detection capillary [17].

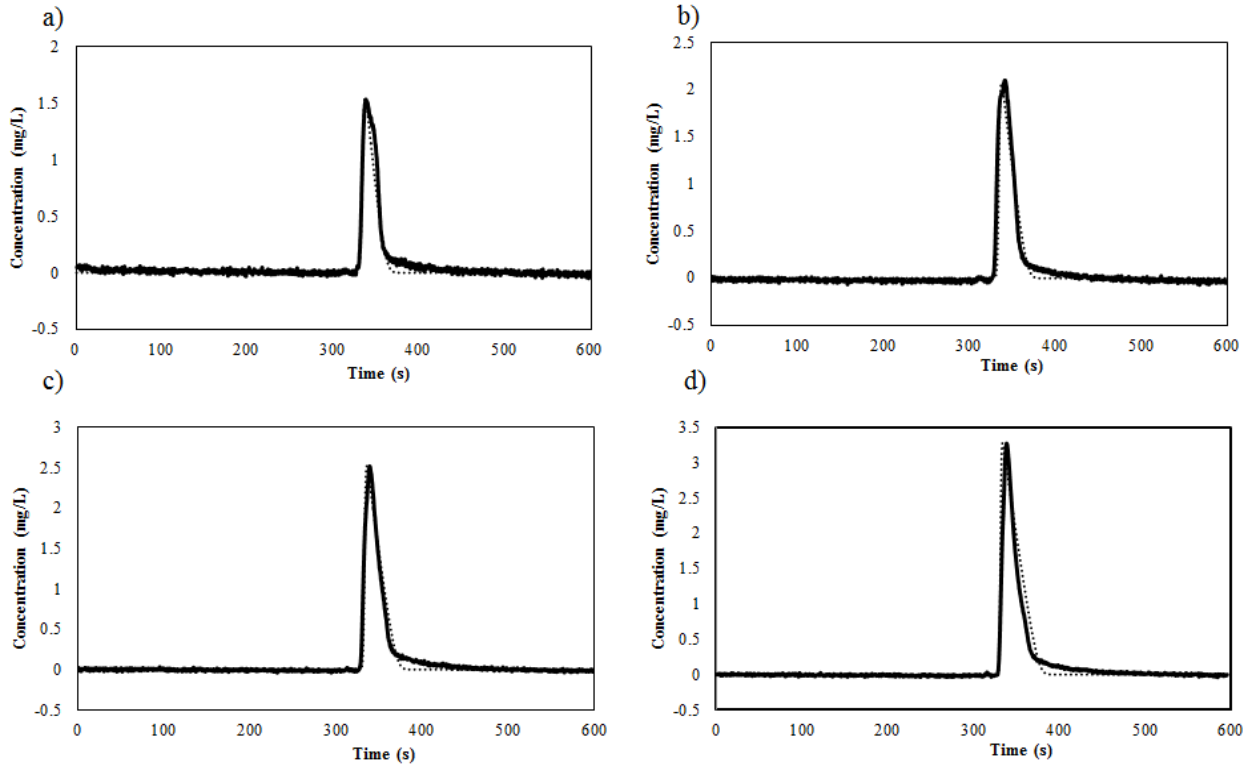

Fig. 5. Experimental (solid lines) and calculated band profiles (dotted lines) of fenoprofen on NPAC. a) 100 mg/L b) 150 mg/L c) 200 mg/L d) 300 mg/L. Experimental conditions are the same as in Fig. 3. The best isotherm parameters are listed in table 2.

The validity of the selected isotherm model in case of naproxen was also checked by comparing calculated and experimental band profiles (Fig. 6). As can be seen, the naproxen band profile on NPAC is well predicted by the anti-Langmuir model in the whole concentration range. The observed minor deviation at the peak rear can again most probably be attributed to coupling dispersion.

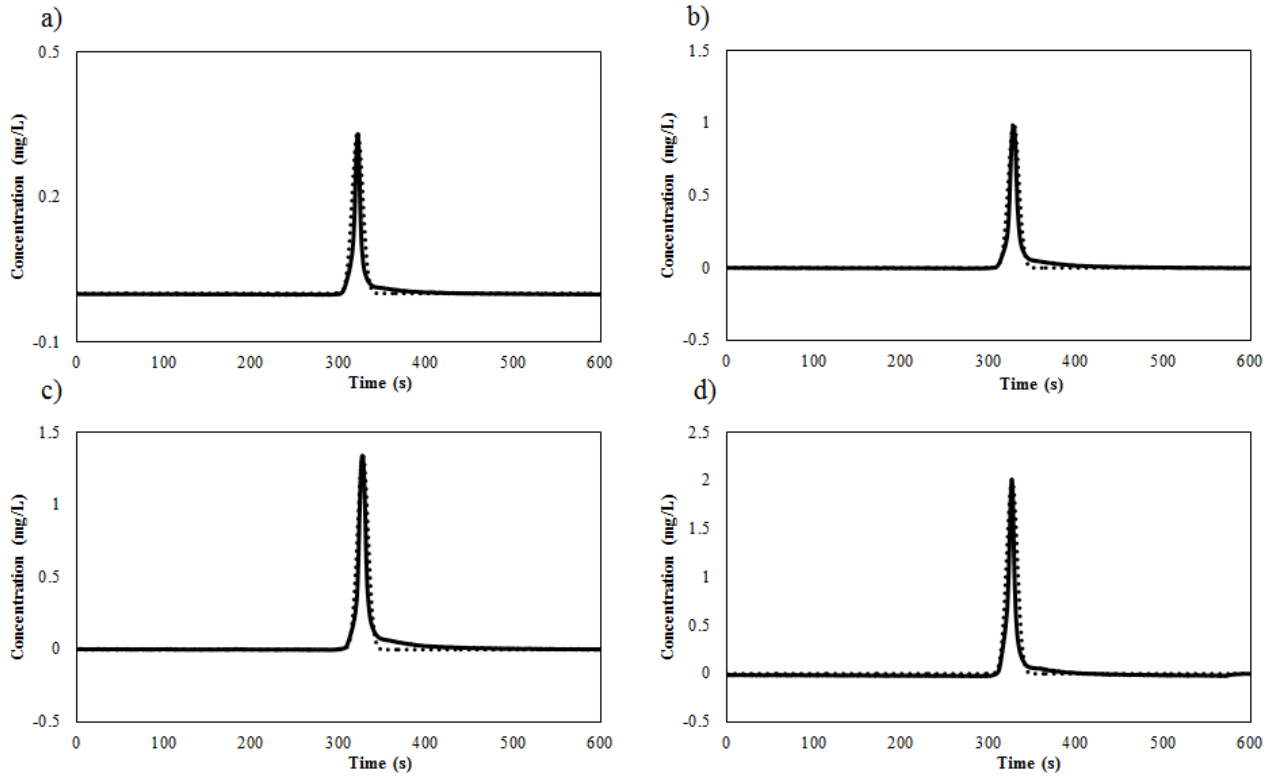

Fig. 6. Experimental (solid lines) and calculated band profiles (dotted lines) of naproxen on NPAC a) 25 mg/L b) 75 mg/L c) 100 mg/L d) 150 mg/L. Experimental conditions are the same as in Fig. 4. The best isotherm parameters are listed in table 3.

4.5. The column efficiency in overloaded condition

For a linear isotherm the plate number is independent of the sample concentration. The elution bands studied here are operated in nonlinear condition at overloading conditions, with the peak

width determined by the isotherm curvature [35]. As expected, the column efficiency decreases with increasing concentration. The obtained result for naproxen has been showed in Fig. 7a, showing a modest sensitivity to concentration. A much might higher sensitivity was observed for fenoprofen (Fig.7b).

Using predictable band shapes, one can also assess the separation performance for given conditions. To this end, the plate heights of the calculated peaks calculated, and subsequently compared to the experimentally obtained values. As is shown in Fig.7, an excellent agreement can be observed in the whole concentration range of both analytes, except 25 ppm concentration, which indicates the selected isotherm models can describe the NAPC behavior well.

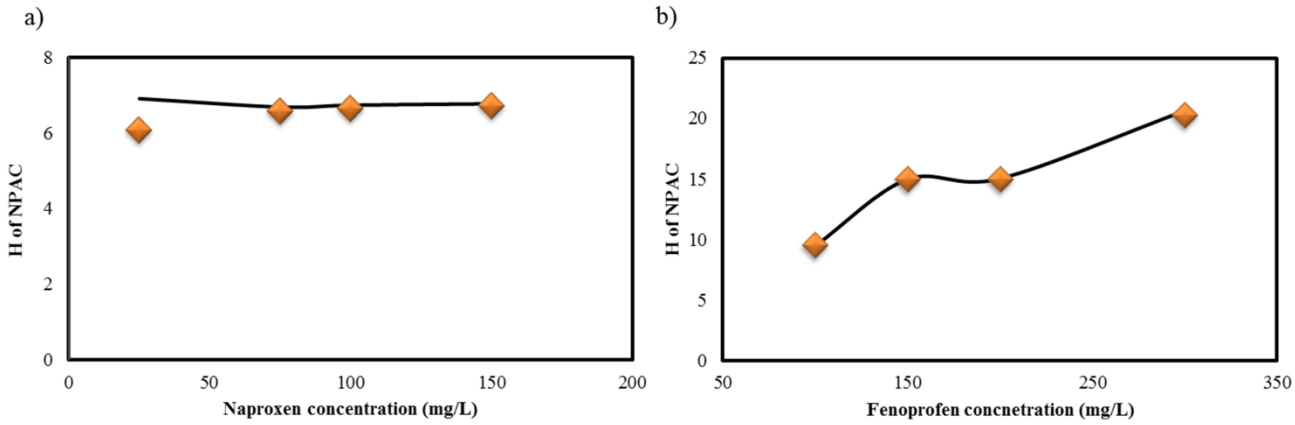

Fig. 7. Plate height as a function of analyte concentration calculated using experimentally obtained (points) and calculated values (solid lines) using the method of moments for a) naproxen b) fenoprofen. Experimental conditions are the same as in Fig.2.

5. Concluding remarks

In liquid chromatography, the adsorption isotherms offer a picture of the process occurring inside the column. These are key importance at a preparative and production scale, therefore, in this study the parameters of the adsorption equilibrium isotherms on NPAC using naproxen and fenoprofen as two model compounds were determined. The adsorption mechanism of naproxen is well described by the anti-Langmuir isotherm model. The adsorption of fenoprofen exhibited a Langmuirian behavior. Injection of naproxen to the NPAC on the overloaded condition led to a fronting peak, however, injection of high concentration of fenoprofen created a tailing peak. The

band shape and isotherm model is determined based on the analytes and the stationary phase as well as mobile phase. In the selected mobile phase, naproxen showed that solute–solute interaction more than solute-stationary phase interaction. As a result, naproxen eluted with a fronting peak and its behavior on NPAC is in line with the anti-Langmuir isotherm model. On the contrary, fenoprofen showed a higher tendency to interact with NPAC surface, resulting in Langmuirian behavior and a tailing peak. The band profiles and plate heights can be well predicted for overloading conditions. The excellent agreement between experimental and predicted results for both analytes indicates that the selected isotherm models are well suitable.

Acknowledgments

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Conflicts of interest

Wim De Malsche is (co-)founder and shareholder of PharmaFluidics, a company that commercializes pillar array columns.

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